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Phenotypic and Genotypic Characterization of Indigenous *Lactobacillus* Community from Traditional Istrian Ewe's Cheese

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Summary

The objective of this study is to describe the diversity of indigenous cultivable community of the lactobacilli associated with the production of traditional Istrian cheese and to get a collection of well characterized strains. Raw milk and cheese samples were collected from three different farms in Istria during ripening. A total of 212 mesophilic and thermophilic *Lactobacillus* isolates as well as bulk colonies (consortia) were investigated using culture-dependent approach combining phenotyping and genotyping. Biochemical fingerprinting with PhenePlate-LB system preliminary grouped 212 isolates in 16 distinct PhP types. Only one representative isolate from each PhP cluster was further analyzed by genotyping for a reliable identification at the genus and species level by employing PCR techniques and sequencing of 16S rRNA genes. Sequence analysis of 16S rRNA revealed the presence of *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus casei*, *Lactobacillus paracasei* and *Lactobacillus rhamnosus*. Lactobacilli were screened for possible resistance against seven selected antibiotics: ampicillin, tetracycline, penicillin, rifampin, clindamycin, erythromycin and vancomycin. Although there was no clear pattern of antimicrobial susceptibility to most tested antibiotics, all representative isolates were resistant to vancomycin. The analysis of bulk colonies by denaturing gradient gel electrophoresis (DGGE) identified *Lactobacillus plantarum* and *Lactobacillus brevis* as predominant members of *Lactobacillus* population. *Pediococcus pentosaceus*, *Pediococcus acidilactici*, *Streptococcus* sp. and *Leuconostoc mesenteroides* were also detected as part of the analysed consortia. The prevalence of identified species and community members of lactobacilli agrees with other studies of raw milk cheese and represents a useful base for further selection.

Key words: Istrian cheese, lactobacilli, PCR, PCR-DGGE, sequencing

Introduction

The microbial flora of different cheese types, particularly those made from raw milk is very complex. Predominant inhabitants of such microbiota of raw milk cheese are nonstarter lactic acid bacteria (NSLAB) composed of many strains important for cheese ripening and flavour development (*1,2*). The presence of NSLAB in the cheese matrix can be a result of contamination during

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the manufacturing or they can come from raw milk. As raw milk cheese has more intense and typical flavour in comparison with pasteurized milk cheese, the interest in studying structural and functional diversity of the NSLAB during the raw milk cheese ripening has been increased (*3–5*). Lactobacilli constitute a significant proportion of NSLAB communities of raw milk cheese and most frequently encountered are facultatively heterofermentative lactobacilli (FHL) belonging to the species: *L. casei*, *L. paracasei*, *L. plantarum*, *L. brevis*, *L. rhamnosus* and *L. curvatus* (*6–8*). Heterogeneity of NSLAB strains with unique and diverse properties represents a key factor for improving authenticity of traditional cheese, when compared to commercially available starter strains.

The bacteria belonging to *Lactobacillus* genus have been widely studied among artisanal cheese types (*3,7, 9–11*). Molecular-based methods and development of primers and probes that target genes coding for the 16S and 23S rRNA have allowed precise description and identification of *Lactobacillus* strains that dominate the fermentation process and ripening of raw milk cheese (*12–15*).

The current study was initiated to identify prevailing strains of lactobacilli present in traditional Istrian cheese towards preserving its specificity and authenticity. Istrian cheese is traditional Croatian product made from raw ewe's milk of autochthonous Istrian breed. It is hard, ripened for three to four months, and produced without the addition of selected starter cultures (*16*). The traditional cheese making process at the farmhouse level has remained unchanged over time, resulting in typical cheese features of the local region.

The aim of this study is to obtain the most complete description of the cultivable autochthonous lactobacilli isolated from ewe's cheese traditionally manufactured in the Istrian region of Croatia. The work represents a combined approach of phenotypic and genotypic characterization of randomly isolated lactobacilli by employing Phene Plate-LB system, genus- and species-specific PCR assays and sequencing of 16S rRNA genes. Additionally, the evaluation of overall *Lactobacillus* community collected from cells in bulk (consortia) by using PCR-DGGE has been performed. Susceptibility to clinically relevant antibiotics was investigated as well, regarding the fact that food lactic acid bacteria, including lactobacilli, may act as reservoirs for antibiotic-resistance genes. This comprehensive overview of dominant strains and their diversity in Istrian cheese is the first step towards its selection, preservation and introduction for technological assessment as starter culture candidates.

Materials and Methods

Sampling

Milk and cheese samples were collected from three different farms (F1, F2 and F3) in the region of Istria in Croatia. Milk and fresh cheese were aseptically sampled at day zero; cheese samples were collected after 30, 60, 90 and 120 days of ripening.

Bacterial isolation, enumeration and collection of cells in bulk (consortia)

A mass of 10 g of cheese samples was homogenized in 90 mL of sterile saline solution for 3 min in a blender BagMixer® 400 (Interscience, St. Nom, France). Ten-fold serial dilutions of ewe's milk and cheese homogenates in sterile saline solution were used for microbial enumeration. Two series of de Man-Rogosa-Sharpe agar (MRS, Merck, Darmstadt, Germany) plates were inoculated and incubated at 25 and 42 °C for 48 h anaerobically using a Genebox system (bioMérieux, Marcy l'Etoile, France). Bacterial counts of mesophilic and thermophilic lactobacilli were obtained after incubation and expressed as log CFU/g of cheese or log CFU/mL of milk. Colonies were randomly picked from plates of each batch of cheese or milk samples and subcultured to purity at least twice on MRS agar plates and stored at –80 °C in MRS medium containing 15 % (by volume) glycerol. After microbial counts, plates were used for collection of bulk colonies (consortia). Bulk colonies from MRS plates of the highest dilutions of each raw milk and cheese samples obtained from F1 and F2 were harvested and suspended with quarter strength of Ringer's solution. A volume of 2 mL of each suspension of cells was used for DNA extraction.

Phenotypic characterization and biochemical fingerprinting

All isolates were tested for Gram stain and catalase production. The catalase test was performed by using 3 % H_2O_2. Catalase-negative and Gram-positive bacilli were subjected to a biochemical fingerprinting with PhenePlate™ system modified for typing lactobacilli (PhP-LB) as described by Čanžek Majhenič *et al.* (*9*). PhP fingerprints of the isolates were compared pairwise and similarity between each pair was expressed as the correlation coefficient. The similarity matrix was clustered according to the unweighted pair-group method by using average linkages (UPGMA) to construct a dendrogram. All calculations of correlation coefficients and clustering were performed with software package PhPWIN (PhPlate AB, Stockholm, Sweden).

Antibiotic susceptibility testing

The antibiotic susceptibility of representative lactobacilli isolates was determined with the disc diffusion test (BBL™ Sensi-Disc™ Antimicrobial susceptibility Test Discs; Becton, Dickinson and Company, Le Pont de Claix, France) with the following discs: ampicillin 2 μg (BD 231263), tetracycline 5 μg (BD 231343), penicillin 10 U (BD 231321), rifampin 5 μg (BD 231544), erythromycin 15 μg (BD 254731) and clindamycin 10 μg (BD 254733). The guidelines for disc diffusion method had been provided by the National Committee for Clinical Laboratory Standards (NCCLS). The antibiotic susceptibility tests were performed on MRS agar, instead of Mueller Hinton base medium (*17*) according to the protocol described by Čanžek Majhenič *et al.* (*9*). Additionally, susceptibility of the PhP representatives to vancomycin was analysed with E-test method (AB Biodisk, Solna, Sweden) to determine minimum inhibitory concentration (MIC). MICs were determined in MRS agar with the same protocol

implemented for disc diffusion method. The measured inhibition zone diameters were interpreted as resistant, intermediate or sensitive in accordance with the NCCLS (*18*).

Genotypic characterization

DNA extraction from isolates

Genomic DNA from PhP representative isolates of lactobacilli and reference strains (*L. brevis* LMG 6906^T, *L. casei* LMG 6904^T, *L. plantarum* LMG 9206, *L. paracasei* ssp. *paracasei* DSM 5622) was extracted from overnight MRS cultures using DNeasy® Tissue kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The quantity of DNA extracts was checked with a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, USA).

DNA extraction from bulk colonies (consortia)

Consortia of all raw milk and cheese samples obtained from F1 and F2 were centrifuged at 3500 rpm for 10 min. Pellets were resuspended in 500 μL of TE buffer (10 mM Tris, 1 mM EDTA, pH=8) containing 20 mg/mL of lysozyme and 10 μL of mutanolysin (1 U/μL) (Sigma-Aldrich, Darmstadt, Germany) and incubated for 2 h at 37 °C. The lysed samples were vortexed and transferred to predispensed reagent cartridges. Total DNA from the consortia of all raw milk and cheese samples was extracted and purified with Maxwell® 16 Tissue DNA Purification Kit (Promega, Madison, WI, USA) using Maxwell® 16 automated sample purification system (Promega) as recommended by the manufacturer.

PCR amplification

To determine the affiliation to genus level of PhP representative isolates, genus-specific PCR assay was carried out with *Lactobacillus* genus-specific primer set LbLMA1--rev (5'-CTCAAAACTAAACAAAGTTTC-3') and R16-1 (5'-CTTGTACACACCGCCCGTCA-3') designed by Dubernet *et al*. (*14*). The PCR reaction mixture and amplification conditions were applied under the conditions described by Dubernet *et al.* (*14*). The representative isolates were additionally amplified by using universal bacterial primer sets F968 (5'-AACGCGAAGAACCTTAC-3') and gc R1401 (5'-CGGTGTGTACAAGACCC-3') designed by Nübel *et al.* (*19*). The PCR mixture and amplification program were previously described by Nübel *et al.* (*19*). The PCR products were separated on 1.5 % agarose gels (100 V, 1 h), stained with ethidium bromide and visualized under a UV transilluminator. The obtained PCR amplicons were purified using MiniElute PCR purification kit (Qiagen, Hilden, Germany) as recommended by the manufacturer, and sequenced.

Species-specific PCR

Species-specific PCR was used for identification of different species within the genus *Lactobacillus* and for precise verification of the results of 16S rRNA gene sequence analysis. According to the preliminary species identification by 16S rRNA gene sequencing, the following combinations of species-specific primers were used in subsequent PCR reactions: LowLac/Plant1 for *L. plantarum* (*15*), BrevI/BrevII for *L. brevis* (*20*), casei/Y2 for *L. casei*, rham/Y2 for *L. rhamnosus*, para/Y2 for *L. paracasei* (*21*). The PCR mixtures and amplification programs had previously been described as follows: *L. plantarum* by Chagnaud *et al.* (*15*), *L. brevis* by Guarneri *et al.* (*20*), *L. casei*, *L. paracasei* and *L. rhamnosus* by Ward and Timmins (*21*).

Profiling of *Lactobacillus* community (consortia)/PCR-DGGE analyses

Bulk DNA extracted from the consortia of lactobacilli from raw milk and cheese samples from F1 and F2 were subjected to PCR-DGGE. The V3 region of 16S rRNA genes was amplified using universal bacterial primer sets GC338f (5'-ACTCCTACGGGAGGCAGCAG-3') and 518r (5'-ATTACCGCGGCTGCTGG-3') as previously described by ben Omar and Ampe (*22*). In order to increase the specificity of the amplification, a 'touchdown' PCR was performed and programmed as follows: after initial denaturation of DNA at 94 °C for 5 min, 30 cycles of 95 °C for 1 min, *55 °C for 1 min (*annealing temperature was gradually decreased from 65 to 55 °C by 2 °C each two PCR cycles) and 72 °C for 3 min were performed. Final extension was carried out at 72 °C for 10 min. The PCR products were analyzed by denaturing gradient gel electrophoresis using Bio-Rad D-code apparatus (Bio-Rad Laboratories, Hercules, CA, USA) as described previously by Muyzer *et al.* (*23*). Optimal separation of PCR products was performed in 8 % (by mass per volume) polyacrylamide gels (ratio of acrylamide and bisacrylamide 37:1) containing a 40 to 60 % urea-formamide denaturing gradient. Electrophoresis was run for 6 h at 120 V and 60 °C. The gels were stained for 20 min in 1×TAE containing 1×SYBR Green (Sigma-Aldrich) and photographed under UV illumination. Different DGGE bands with identical migration profiles were selected, excised from the gel and purified in water as described by Cocolin *et al.* (*24*). Eluted DNA of each DGGE band was reamplified by using the above described primers without GC clamp and the conditions described above. Reamplified PCR products were purified with a MinElute PCR Purification Kit (Qiagen) as recommended by the manufacturer, and sequenced.

16S rDNA sequence analyses

Sequencing was performed by DNA service unit of Ruđer Bošković Institute, Zagreb, Croatia. The obtained sequences were aligned and compared with the sequences deposited in the GenBank database (National Center for Biotechnology Information, Bethesda, MD, USA) using the BLAST algorithm.

Nuceotide sequence accession numbers

The sequences were deposited in the GenBank database with the accession numbers from JF833435 to JF833450.

Results and Discussion

Isolates and viable counts

Two hundred and twelve isolates of presumptive lactobacilli were isolated from all investigated samples of raw milk and cheese. Viable counts of mesophilic and thermophilic lactobacilli of raw milk and cheese during ripening from three different farms are shown in Table 1. The viable counts of mesophilic and thermophilic lactobacilli in raw milk samples were relatively low in

Table 1. Bacterial counts of the lactobacilli in milk (M) and cheese (Ch) samples from three different farms (F1–F3) during 120 days of ripening

Farm	Sample	*t*(ripening)	Bacterial counts/(log CFU per g of cheese or log CFU per mL of milk)	
		day	Mesophilic lactobacilli	Thermophilic lactobacilli
F1	M1	0	2.04	1.62
	Ch1	0	6.22	3.28
	Ch1(1)	30	6.86	5.22
	Ch1(2)	60	7.07	5.93
	Ch1(3)	90	6.21	5.32
	Ch1(4)	120	4.93	6.16
F2	M2	0	2.08	1.00
	Ch2	0	5.38	4.62
	Ch2(1)	30	5.42	5.10
	Ch2(2)	60	6.01	6.48
	Ch2(3)	90	5.96	6.52
	Ch2(4)	120	5.35	6.41
F3	M3	0	1.95	n.d.
	Ch3	0	4.98	n.d.
	Ch3(1)	30	6.56	n.d.
	Ch3(2)	60	6.61	n.d.
	Ch3(3)	90	6.42	n.d.
	Ch3(4)	120	5.34	n.d.

n.d.=not detected; mesophilic lactobacilli: MRS, anaerobic growth at 25 °C; thermophilic lactobacilli: MRS, anaerobic growth at 42 °C

comparison with the counts of lactobacilli among cheese samples during ripening. In some samples of raw milk, the number of lactobacilli was at the detection limit. Mesophilic and thermophilic lactobacilli reached the highest counts during the mid-stage of ripening with maximum values between 6.01 and 7.07 log CFU/g, and 6.16 and 6.52 log CFU/g, respectively. The abundance of mesophilic lactobacilli was slightly higher among all investigated samples. The obtained values mostly persisted or slightly decreased until the end of ripening period, which is in agreement with the counts found in other ewe's milk cheese samples (*9*,*25*). The thermophilic flora was not detected at F3.

Phenotypic characterization

All 212 Gram-positive and catalase-negative presumptive lactobacilli were differentiated into preliminary groups based on carbohydrate fermentation assays specified for typing lactobacilli (PhP-Lb). Data were obtained after three consecutive readings of growth on ready-made microtiter plates with 24 different substrates and analysed with PhenePlate™ software. The biochemical patterns of all isolates were compared and clustered using UPGMA (Fig. 1). All isolates were run in the same intra-assay and ID level was 0.975. Isolates with higher level of similarity than the ID level were assigned to the same PhP type (*26*). Based on the cluster analysis, all of 212 mesophilic and thermophilic lactobacilli were distributed into 16 PhP types. The representative of each PhP type was selected and further analyzed (Table 2). Results showed high phenotypic variations and diversity among the isolates although most of the isolates gave unique phenotypes denoted as single isolates (Si). Such biodiversity is not surprising knowing that autochthonous microbiota from traditional food have specific genetic reservoir, which should be preserved and investigated in detail (*9*,*27*,*28*). Diversity among *Lactobacillus* population may contribute to subsequent desirable fermentative reactions, reflecting specific sensory properties and authenticity of Istrian cheese. The PhP-Lb system was used primarily for screening of biodiversity of indigenous lactobacilli as well as a method for reducing their number for following genetic analyses. However, a great number of Si indicates that PhP system alone cannot serve as a method for determining relationships between strains in such complex ecological studies (*29*).

Each of the PhP type was screened for possible resistance against seven selected antibiotics using agar disc diffusion and E-test methods, the results of which are presented in Table 3. It was found that lactobacilli were sensitive to erythromycin (81.25 %), clindamycin (68.75 %) and rifampin (56.25 %). Furthermore, high number of isolates was grouped as resistant to ampicillin (87.5 %), tetracycline (81.25 %) and penicillin (68.75 %), which supports the fact of intrinsically coded resistance to certain antibiotics among lactobacilli (*6*). However, the results obtained in our study indicate that there was not a clear pattern for antimicrobial resistance among the tested strains suggesting species-dependant antibiotic resistance occurrence (*30*). The results of E-test method revealed vancomycin resistance of all representative lactobacilli. Although strains of strictly homofermentative lactobacilli are known to be sensitive to vancomycin, Bernardeau *et al.* (*6*) reported that *L. plantarum*, *L. casei*, *L. salivarius*, *L. leishmanii*, *L. acidophilus*, as well as pediococci and *Leuconostoc* spp. have a high natural resistance to this antibiotic. Antibiotic resistances of food lactic acid bacteria represent a great concern since lactic acid bacteria may act as a vehicle of antibiotic resistance genes (*30*), which needs further investigations.

Genotypic characterization

Genus- and species-specific identification

A primer pair LbLMA1-rev/R16-1 was chosen as most suitable for identification of strains related to genus *Lactobacillus* (*12*,*14*). All of 16 PhP-LB representative strains gave a specific PCR product approx. 250 bp long. Additionally, DNA of representative isolates was amplified with 968F and 1401R primers and amplicon of 433 bp was generated. The PCR products of both amplifications were used for sequencing for identification at the species level and to check the primer specificity for *Lactobacillus* genus. Among 16 representatives, the sequencing identified 15 strains as *Lactobacillus* members (Table 2). Nine sequences were related to *L. plantarum*, four to *L. brevis*, one to *L. casei* and one sequence was interpreted as *L. casei*, *L. paracasei* or *L. rhamnosus*. One isolate was identified as *Pediococcus pentosaceus* (Table 2). As applied primer pair LbLMA1-rev/R16-1 showed low specificity in determining closely related genera, it was necessary to supplement the study with more accurate assays such as 16S rRNA sequencing or species-specific PCR.

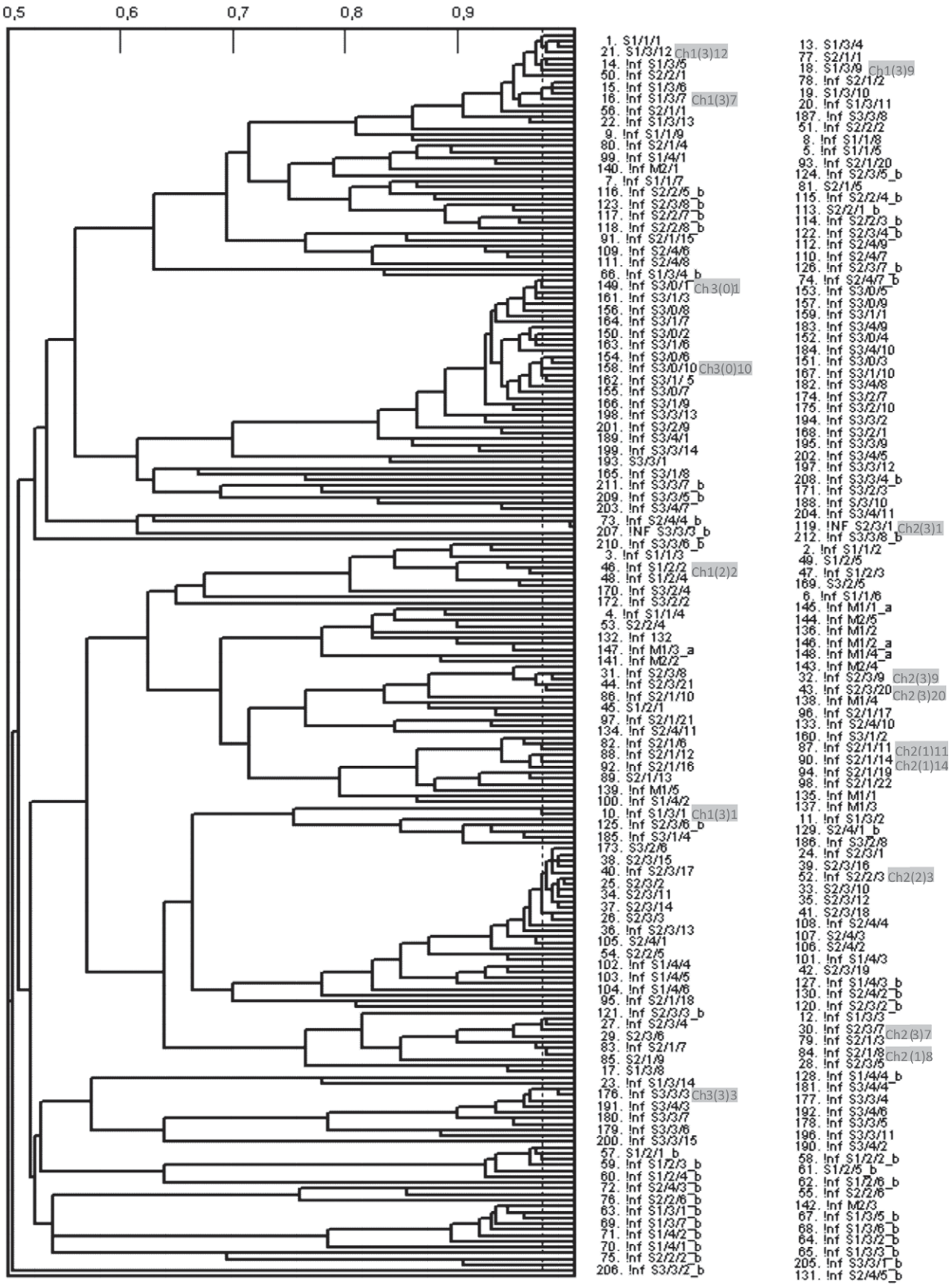

Fig. 1. Dendrogram derived from UPGMA clustering of the PhenePlate typing data of 212 presumptive lactobacilli isolates collected from milk and cheese samples from three different farms during ripening. Sixteen PhP representative isolates are highlighted

Table 2. Phenotypic and genotypic identifications of PhP representatives

PhP representative	PhP type	PCR identification	Taxonomic identification of 16S rRNA gene amplicons	Acc. no.
Ch1(3)12	1	*L. casei*	*L. casei*	JF833439
Ch1(3)1	2	*L. casei*	*L. rhamnosus*, *L. casei*, *L. paracasei*	JF833450
Ch1(3)9	3	*L. plantarum*	*L. plantarum*	JF833438
Ch1(3)7	4	*L. brevis*	*L. brevis*	JF833449
Ch2(2)3	5	*L. plantarum*	*L. plantarum*	JF833446
Ch2(3)/7	6	*L. plantarum*	*L. plantarum*	JF833447
Ch2(3)9	7		*P. pentosaceus*	JF833441
Ch2(3)20	8	*L. plantarum*	*L. plantarum*	JF833442
Ch1(2)2	9	*L. brevis*	*L. brevis*	JF833437
Ch2(1)8	10	*L. plantarum*	*L. plantarum*	JF833443
Ch2(1)11	11	*L. plantarum*	*L. plantarum*	JF833444
Ch2(1)14	12	*L. plantarum*	*L. plantarum*	JF833445
Ch2(3)1	13	*L. brevis*	*L. brevis*	JF833440
Ch3(0)1	14	*L. plantarum*	*L. plantarum*	JF833436
Ch3(0)10	15	*L. brevis*	*L. brevis*	JF833435
Ch3(3)3	16	*L. plantarum*	*L. plantarum*	JF833448

Acc. no.=accession number in GenBank

Table 3. Antibiotic susceptibility profiles of PhP representative isolates

PhP representative	Antimicrobial agents						
	Disc diffusion test						E-test
	Erythromycin	Rifampin	Ampicillin	Penicillin	Tetracycline	Clindamycin	Vancomycin MIC/(μg/mL)
Ch1(3)12	S	S	S	S	R	S	R≥256
Ch1(3)1	S	S	R	R	R	S	R≥256
Ch1(3)9	S	S	S	S	R	S	R≥256
Ch1(3)7	S	S	R	S	R	R	R≥256
Ch2(2)3	S	I	R	R	S	I	R≥256
Ch2(3)7	S	I	R	R	R	I	R≥256
Ch2(3)9	S	R	R	R	R	S	R≥256
Ch2(3)20	I	R	R	R	R	R	R≥256
Ch1(2)2	S	S	R	S	I	S	R≥256
Ch2(1)8	I	R	R	R	R	S	R≥256
Ch2(1) 11	I	R	R	R	R	I	R≥256
Ch2(1)14	S	S	R	R	R	S	R≥256
Ch2(3)1	S	S	R	R	R	S	R≥256
Ch3(0)1	S	S	R	R	R	S	R≥256
Ch3(0)10	S	I	R	R	I	S	R≥256
Ch3(3)3	S	S	R	S	R	S	R≥256

S=sensitive, I=intermediate, R=resistant

In order to confirm the 16S rRNA gene sequencing results and to resolve the ambiguities associated with isolate Ch1(3)1, which had no clearly defined affiliation according to the 16S rRNA sequence analyses, species-specific PCR was applied. By application of species-specific primers casei/Y2 for *L. casei* and para/Y2 for *L. paracasei*, the obtained PCR product resolved ambiguities and identified the isolate as *L. casei* (Fig. 2). No PCR product was observed when *L. paracasei* primer pair was used. Species-specific PCR primers based on the 16S-23S rRNA intergenic spacer regions were valuable in confirming sequence identification of Php representative isolate Ch1(3)1, particularly because it is difficult to reach a clear-cut taxa of *L. casei* group of isolates (*L. casei*/*L. paracasei*/*L. rhamnosus*) (7).

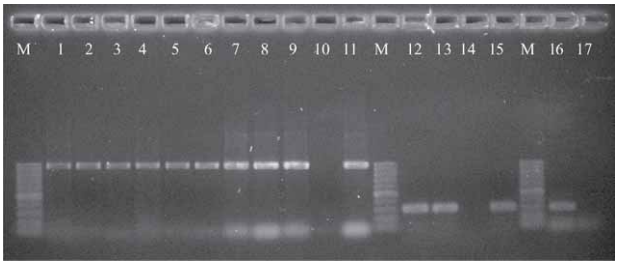

Fig. 2. Species-specific PCR products of Php representative lactobacilli generated with *L. plantarum*-specific primers. Lane M: 100 bp DNA Ladder; lanes 1–9: (Ch1(3)9, Ch2(1)8, Ch2(1)11, Ch2(1)14, Ch2(2)3, Ch2(3)7, Ch2(3)20, Ch3(0)1 and Ch3(3)3); lane 10: negative control; line 11: positive control *L. plantarum* LMG 9206; lane M: 100 bp DNA Ladder; *L. casei*-specific primers: lanes 12 and 13: Ch1(3)1 and Ch1(3)12; lane 14: negative control; lane 15: positive control *L. casei* LMG 6904^T; lane M: 100 bp DNA Ladder; *L. paracasei*-specific primers: lane 16: positive control *L. paracasei* ssp. *paracasei* DSM 5622; lane 17: Ch1(3)1

L. brevis-specific primers were used for amplification of 4 isolates and PCR product of 1340 bp was generated with all four tested strains, which is in agreement with the results of sequencing (Fig. 3). The results of sequencing for 9 strains identified as *L. plantarum* (Table 2) were confirmed with specific-primes for *L. plantarum* by generating PCR product of 996 bp (Fig. 2). However, results of 16S rRNA gene sequencing supplemented with species-specific PCR confirmed that *L. plantarum* was the predominant isolate of all PhP representatives.

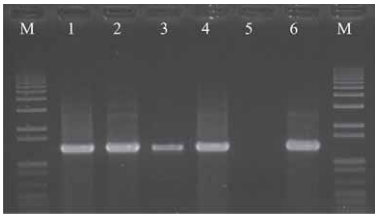

Fig. 3. Species-specific PCR of PhP representative lactobacilli generated with *L. brevis*-specific primers. Lane M: 1 kb plus DNA Ladder, lane 1: Ch1(3)7, lane 2: Ch1(2)2, lane 3: Ch2(3)1, lane 4: Ch3(0)10, lane 5: negative control, lane 6: positive control *L. brevis* LMG 6906, lane M: 1 kb plus DNA Ladder

PCR-DGGE fingerprinting of microbial consortia

Total DNA extracted from mesophilic and thermophilic consortia of lactobacilli from all samples collected from F1 and F2 were subjected to PCR-DGGE analyses.

DGGE fingerprints derived from PCR amplification of V3 16S rRNA gene region showed complex profiles and revealed remarkable polymorphism among the analyzed samples. Fig. 4 shows representative DGGE profiles of 16 microbial consortia collected from two farms (F1 and F2). The variability of DGGE patterns was assessed by number of the bands and migration distances. In general, higher numbers of bands were found in mesophilic consortia of all cheese samples from F1 and F2 farms, suggesting considerable complex microbiota. DGGE profiles from F2 cheese samples were different compared to profiles from F1 cheese, although similar patterns were also observed. The variability among some samples could be a result of differences in NSLAB population typical for each farm. The DGGE profiles were quite consistent in samples of F2 cheese from day 0 until 120 days of ripening. The selected DGGE bands were excised, reamplified, purified and sequenced. Partial 16S rRNA gene sequences obtained from dominant selected bands were compared to those available in the GenBank database and the closest known relative species was determined. The results of sequencing and data analysis are shown in Table 4. The predominant species in all samples from farm F2 corresponded to *Lactobacillus plantarum* (band d) and *Lactobacillus brevis* (band e); moreover, they were also detected as predominant at some ripening stages among cheese samples from F1. This result could correlate with the results obtained from single isolate identifications where *L. plantarum* was the dominant species on farm 2. Both species usually occur in high number in artisanal cheese ecosystems. *Lactobacillus plantarum* is recognized as contributing to flavour development (*5,31*) and as species with antibacterial ability, producing plantaricin C (*32*), while *L. brevis* has been hypothesized to impact cheese ripening with its potential to metabolise amino acids *via* transaminating reactions and endogenous transamination (*11,33*). Among the presumed species of the genus *Lactobacillus*, sequence analyses revealed *Pediococcus pentosaceus*, *Pediococcus acidilactici*, *Streptococcus* sp. and *Leuconostoc mesenteroides* as species present in bulk, which indicates non-selectivity of MRS medium (*34*). Neverthe-

Table 4. Bacterial species identification after sequencing V3 16S rRNA fragments from DGGE gel of microbial consortia

Samples	Bands[#]	Taxonomic identification of 16S rRNA gene amplicons	Acc. no.
Ch2(1), Ch2(3), Ch2(2)*	d	*L. plantarum*	FJ749579.1
CH1(1)*, Ch1(3)*	d	*L. plantarum*	GU430776.1
Ch1(1)*, Ch2(1), Ch2(3), Ch2(2)*, Ch2(2) Ch2(3)*,Ch2(4)*	e	*L. brevis*	HM162416.1
M2	f	*Leuconostoc mesenteroides* ssp. *mesenteroides*	HM059009.1
Ch1(1)*, Ch1(3)* M2, Ch1(2)*	c	*Pediococcus acidilactici*	GQ240304.1
Ch1(3)*, Ch2(1)*	a	*P. pentosaceus*	HM536141.1
Ch1(4)	g	*Streptococcus* sp.	GQ482946.1
Ch1(3)* Ch1(2)*		*Leuconostoc mesenteroides* ssp. *mesenteroides*	HM562987.1
Ch2(1)*, Ch1(3)* Ch2(3)*	b	*Lactobacillus acidipiscis*	AB326356.1

[#]band identification according to Fig. 4.
Acc. no.=accesssion number in GenBank
*samples of milk and cheese marked with asterisk indicate mesophilic consortia (anaerobic growth at 25 °C) and samples without asterisk indicate thermophilic consortia (anaerobic growth at 42 °C)

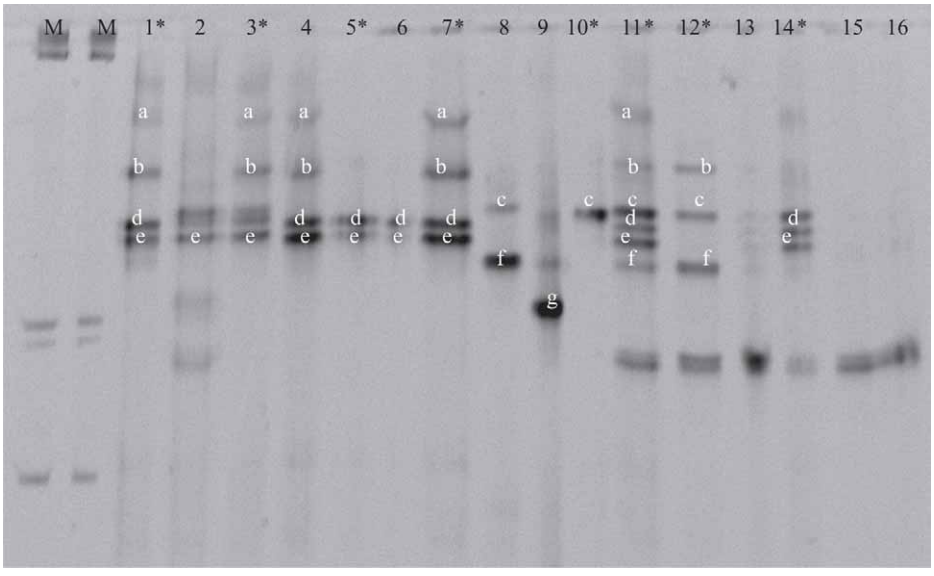

Fig. 4. DGGE fingerprinting profiles of amplified 16S rRNA V3 region of bulk DNA extracted from microbial consortia overgrowth on MRS agar of milk (M) and cheese Ch(0), Ch(1), Ch(2), Ch(3) and Ch(4) samples during ripening collected from farm 1 and farm 2. Lanes M: 1 kb DNA Ladder, lanes 1–2: Ch2(4), lanes 3–4: Ch2(3), lanes 5–6: Ch2(2), lane 7: Ch2(1), lane 8: M2, lanes 9–10: Ch1(4), lane 11: Ch1(3), lanes 12–13: Ch1(2), lanes 14–15: Ch1(1), lane 16: Ch1(0). Lanes marked with asterisk indicate samples of mesophilic consortia (anaerobic growth at 25 °C) and lanes without asterisk indicate samples of thermophilic consortia (anaerobic growth at 42 °C)

less, it remains the most common medium used for isolation of lactobacilli (*10*). Members of *Pediococci* (band c) and *Leuconostoc* (band f) were abundant in most samples from F1, whereas at F2 they were not detected. The presence of *Leuconostoc mesenteroides* has already been reported in several different cheese ecosystems and is often found in food matrices (*1,31,35,36*). *Pediococcus pentosaceus* was detected in majority of consortia collected from samples F2, and *Pedicoccus acidilactici* in most samples from F1. The presence of pediococci revealed their significant part in NSLAB communities of traditional cheese, underlining their influence on the development of aromatic components during the ripening of cheese (*37*). The only one band identified as *Streptococcus* sp. (band g) was detected in the consortia of fully matured cheese from farm F1 (Ch1(4)). Recently, different studies have demonstrated the presence of *Streptococcus* sp. in cheese as in other dairy products (*38–40*).

Conclusions

Culture-dependent approach followed by phenotyping and genotyping used in this study provided characterization of dominant lactobacillus population and some subdominant species of other LAB genera present in traditional Istrian cheese. The lactobacillus species identities were confirmed by sequencing or by species-specific PCR. Amplification of V3 region followed by DGGE analyses has enabled differentiation of important LAB strains from consortia and confirmed that they could be used as a practical screening tool for the analyses of individual isolates. The combination of applied methods for identification has shown that *L. plantarum* was a dominant species and *L. brevis* to a lesser extent. The presence of other related LAB genera as *Pediococcus pentosaceus*, *Pediococcus acidilactici*, *Streptococcus* sp. and *Leuconostoc mesenteroides* can be considered as a fundamental factor for the maintenance of the typical features of the investigated cheese. Such complex viable community will be preserved and used in further strain selection process providing functional characterization and better insight into the biochemical and sensory aspects of traditionally produced Istrian cheese.

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